

Antifertility Effects and Dominant Lethal Assays for Mutagenic Effects of DEHP

by John Autian*

Results of past animal studies have indicated the antifertility effects of phthalate esters and additional studies have suggested the potential mutagenic effects at very high doses. A toxicity study in mice has also been conducted in which chronic LD_{50} values were calculated for a group of phthalate esters. For DEHP, for example, the acute LD_{50} was 38.35 ml/kg, but after 10 weeks the value fell to 1.37 ml/kg, suggesting that the ester was producing a cumulative toxicity. A more recent study explored the possibility that DEHP might be a cumulative toxic agent even at lower doses in regard to antifertility and mutagenic effects in mice. Preliminary results indicated that antifertility effects occurred with as little as three subcutaneous doses of 1 ml/kg each. Calculations of mutagenic index revealed a dose-dependent effect but no stoichiometric relationship was established.

Introduction

In the United States, concern for potential health threats from the leaching of phthalate esters used as plasticizers in poly(vinyl chloride) items such as blood bags and tubings dates back to a report published in 1967 by Guess et al. (1). Their studies on blood bags containing ACD solution revealed that the plasticizers, as well as degradation products of the ester, were released to the solution. While the quantity of the released chemicals was extremely small, the question was raised for the safety of these products when administered to ill and debilitated patients. Although notice was taken of this report, neither government nor industry took steps to correct the "contaminated" ACD solution or blood products placed in these PVC bags. Their past history of use and their apparent safety mitigated the need for making changes in the plastic though industry had begun to initiate studies to help improve the PVC formula.

Publication of research by Jaeger and Rubin (2) in 1970, however, resurrected the plastic's safety into a broader domain. These investigators reported the detection of residual amounts of phthalate esters in a number of organs and tissues of deceased

patients who had a recent history of blood therapy from PVC blood bags. From that date, considerable research has taken place on the toxicology of the various phthalates, in particular, di(2-ethylhexyl) phthalate (DEHP). Two major phthalate conferences have also been held; one in 1973 and the present one. Since the initial review of the toxicology of phthalate esters by Autian in 1973 (3) additional reviews have appeared in literature. Yet, after a decade and a half, no resolution of the safety issue has occurred, and the volume of medical devices used in medical applications has increased considerably since that time. The recent findings that DEHP has induced tumors in both rats and mice may become the pivotal point for a regulatory agency such as FDA to ban PVC (with phthalate esters) from specific uses (National Cancer Institute, unpublished report).

It is now well established that DEHP and most other diesters of phthalic acid have been found to have a very low order of acute toxicity. Chronic animal studies performed in the 1950s and 1960s (with the exception of the very recent cancer studies) also suggest that low levels of phthalate esters taken in feed on a daily basis have no significant toxicologic consequence, even though there was some indication of liver involvement at the higher dose levels.

A more recent study on chronic toxicity by Lawrence et al. (4) permitted the calculations of

*Materials Science Toxicology Laboratories, University of Tennessee Center for the Health Sciences, Memphis, Tennessee 38163.

chronic LD₅₀ for a group of phthalate esters. In these experiments, a group of mice was injected IP with a series of doses five days per week. At the end of each week, an apparent LD₅₀ was calculated. This procedure was continued until the apparent LD₅₀ remained constant for three consecutive weeks with a minimum of 10 weeks treatment. Table 1 is a summary of this study and includes the acute LD₅₀, the chronic LD₅₀ and a cumulative toxicity factor (acute/chronic). It will be noted that there is a dramatic cumulative factor for both the di-*n*-octyl and di(2-ethylhexyl) phthalates as compared to the other phthalates. This suggests that, under the experimental conditions, these dioctyl and di(2-ethylhexyl) phthalates do have a marked cumulative effect. For example, DEHP has an acute LD₅₀ of 38.35 ml/kg. Over a 10-week period this value is reduced to 1.37 ml/kg. Presently there is no information which explains this type of response, but it may be that the metabolites of the two esters play a much greater role in the mechanism of action than for the lower molecular diesters; this remains conjecture at this time. It might, however, be pointed out that Kevy et al. (5) did find the presence of DEHP (or its metabolites) in livers of monkeys many months after the last administration of the compound. Most investigators, however, do not support the cumulative theory for DEHP based upon their studies in rodents.

Singh et al. (6) studied the teratogenic effects of eight phthalate esters in rats and noted that all compounds, to various degrees, induced birth abnormalities and fetal deaths. Esters included the following: dimethyl, dimethoxyethyl, diethyl, dibutyl, diisobutyl, butyl carobutoxymethyl, dioctyl and di(2-ethylhexyl) phthalates. Groups of animals were administered the various esters by IP injections on days 5, 10, and 15 of gestation at several dose levels. The most widely used phthalate, di(2-ethylhexyl) phthalate (DEHP), was administered at two dose levels, 5 ml and 10 ml/kg, but fetal abnormalities were noted only at the higher dose level.

Since PVC blood bags generally contain DEHP as the plasticizer, a study was conducted by Lewandowski et al. (7) in which two types of PVC blood bag materials were extracted with rat plasma to give a concentration of DEHP approximately equivalent to the dose a human weighing 60 kg could receive during an exchange transfusion of 21-day-old blood. Several dose levels were used, ranging from 1.4 mg/kg per day to 5.3 mg/kg per day. The various doses were administered to groups of pregnant rats IV from day 6 through day 15 of gestation. No teratogenic effects or adverse consequences were noted in any of the experimental animals as compared to control groups not receiving the DEHP, supporting the contention of the authors that blood products stored in PVC would not present a teratogenic risk to pregnant humans.

In another study by Singh and associates (8), DEHP and dimethoxyethyl phthalate (DMEP) were administered by IP injection to adult male mice, and subsequently these mice, individually, were mated to groups of two adult virgin females. This sequence with new females was repeated weekly for 12 weeks. Between days 13 and 17 of gestation, the pregnant mice were sacrificed and the uterine horns and ovaries exposed. Corpora lutea, total number of implantations, early and late fetal deaths, pre-implantation losses and viable fetuses (litter size) were determined. Interpretations of the data suggested to the authors that the two esters had the potential to act as mutagenic agents as well as to suppress fertility.

Recent Preliminary Studies on Antifertility and Mutagenic Effects of DEHP in Mice

As has already been described, DEHP appears to have an antifertility effect when male mice are first treated with the compound and then subsequently mated with virgin female mice. This response in

Table 1. Chronic toxicity of phthalate esters in mice (IP).^a

Ester	Acute LD ₅₀ (ml/kg)	Chronic LD ₅₀ (ml/kg)	Cumulative toxicity factor (acute/chronic)
Dimethyl	3.35	1.18	2.84
Diethyl	2.87	1.39	2.06
Di- <i>n</i> -butyl	3.41	0.85	4.01
Diisobutyl	3.84	1.87	2.05
Di- <i>n</i> -octyl	67.18	3.09	21.74
Di-2-ethylhexyl	38.35	1.37	27.99
Butyl carobutoxymethyl	6.27	3.04	2.06
Bis(2-methoxyethyl)	3.57	1.41	2.53

^aFrom Lawrence et al. (4). Information summarized from Table 3 in Lawrence's article. For the chronic study, groups of mice were injected IP with a series of doses five days per week. At the end of each week, an apparent LD₅₀ was calculated. This procedure was continued until the apparent LD₅₀ remained constant for three consecutive weeks with a minimum of 10 weeks treatment.

general is dose-related. Results from dominant lethal assays also suggest that at high doses DEHP might be acting as a mutagenic agent.

The possibility that DEHP might be a cumulative toxic agent, even at lower doses, prompted our laboratories to initiate several preliminary experiments to clarify whether a possible cumulative effect can impose both an antifertility effect and mutagenic effects during an 8-week period.

Experimental

In the first series of experiments, male mice were injected with DEHP subcutaneously on days 1, 5 and 10 at dose levels ranging from 1.0 ml/kg to 100 ml/kg. Seven or more mice were used for each dose level. Normal virgin females were mated individually with each treated and control male mouse on day 21 following the first injection. Animals of the two sexes were kept together for four consecutive days and females examined every day for the presence of vaginal plugs as an indication of matings. Detection of the vaginal plug was taken as the first day of gestation. Bred females were sacrificed on days

12-13 of gestation, and uterine horns and ovaries were exposed surgically to determine the number of corpora lutea, implantations, preimplantation losses, early fetal deaths and viable fetuses.

A second series of experiments was also conducted essentially following the previously described procedure. Groups of 10 male mice were treated with DEHP subcutaneously on days 1, 5 and 10 at the following dose levels: 1 ml/kg, 2 ml/kg, 5 ml/kg and 10 ml/kg. A group of untreated mice was used as control. Each male mouse was mated with a fresh virgin female 24 hr after the treatment, followed by a change of females every fifth day up to 21 days and then every week up to 8 weeks. All pregnant females were sacrificed as previously and the corpora lutea, implantations, preimplantation losses, early fetal deaths and viable fetuses recorded.

Results

Table 2 summarizes the number of pregnancies occurring for each dose administered, as well as the number of corpora lutea, implants, preimplantation losses, early fetal deaths and live fetuses. It will be

Table 2. Antifertility and mutagenic trends from injection (SC) of DEHP in groups of male mice at days 1, 5 and 10 and mated with normal females on day 21.^a

Group	Incidence of pregnancy	Number of corpora lutea	Implant	Preimplantation losses	Early fetal deaths	Number of live fetuses
Saline control	14/16 (88) (87.5%)	12.62	10.43	2.19	0.37	10.06
DEHP-treated						
1 ml/kg	5/8 (68) (62.5%)	12.87	7.62	5.25	2.75	4.87
2 ml/kg	3/8 (38) (37.5%)	14.00	5.37	8.63	0.87	4.50
5 ml/kg	3/8 (38) (37.5%)	15.12	4.87	10.25	1.00	3.87
10 ml/kg	3/8 (38) (37.5%)	14.50	4.75	9.75	1.62	3.13
15 ml/kg	2/8 (25)	15.52	3.50	12.02	1.12	2.38
20 ml/kg	0/8 (0)	13.87	— ^b	13.87	— ^b	— ^b
40 ml/kg	1/7 (14) (14.2%)	14.14	1.85	12.29	0.00	1.85
60 ml/kg	0/8 (0)	11.25	— ^b	11.25	— ^b	— ^b
80 ml/kg	1/9 (11) (11.1%)	16.33	1.33	15.00	0.00	1.33
100 ml/kg	0/8 (0)	18.44	— ^b	18.44	— ^b	— ^b

^aData are expressed as mean value per animal for each group.

^bNo pregnancies in this group.

Table 3. Mutagenic index (early fetal deaths/total implants per pregnancy) \times 100.

Time of mating	Control	DEHP-treated			
		1 ml/kg	2 ml/kg	5 ml/kg	10 ml/kg
Day 2	0.43	1.81	5.0	2.99	3.58
Days 6	0.34	0.52	0.43	1.42	0.66
Days 11	0.66	0.86	2.14	2.64	3.51
Days 16	0.36	2.02	2.50	2.08	1.60
Days 21	0.19	0.80	1.60	2.47	4.44
Weeks 4	0.38	1.40	1.22	1.15	2.69
Weeks 5	0.45	1.82	3.33	1.49	3.48
Weeks 6	0.30	0.76	3.05	2.86	3.33
Weeks 7	0.18	2.35	1.75	1.23	1.31
Weeks 8	0.60	1.17	2.20	1.55	1.68

noted that an antifertility effect is apparent even at the 1 ml/kg dose level, though statistical analysis was not performed on these data. The trend of antifertility increases as the dose administered to the male mice increases. Increased early fetal deaths and preimplantation losses also can be noted with the treated animals, the trend generally increasing as the dose administered is increased. Again, the data are suggestive and not definitive under the experimental conditions employed.

Data generated from the second series of experiments, in which treated male mice were mated with virgin female mice at different intervals (from day 2 to 8 weeks), were used to calculate the mutagenic index (early fetal deaths/total implants \times 100). These values are shown in Table 3. A review of the table indicates that the mutagenic index increases as the dose is increased, although again it cannot be said if these increases are significantly different from the control group. The trend, however, is clear; the mutagenic index is higher for treated animals on the average than for the control groups at any of the time periods.

Discussion

Previous studies in this laboratory demonstrated that at high doses (IP), the phthalate esters could act as teratogenic and antifertility agents (6). Results from a dominant lethal assay also suggested that the esters might have mutagenic activity (8). Since DEHP was found to exert a cumulative toxicity effect in mice, these experiments were undertaken to determine antifertility and mutagenic effects of repeated injection of DEHP in mice at various dose levels.

As suggested in Table 2, antifertility effects were noted with as little as three subcutaneous doses of 1 ml/kg each. Calculations of mutagenic index (early fetal deaths/total implants per pregnancy \times 100) have been listed in Table 3 for the various dose levels (1, 2, 5 and 10 ml/kg) over an eight-week period. Even though a dose-dependent effect is apparent, no stoichiometric relationship has been established.

Expression of mutagenic index in terms of per pregnancy excludes any possible antifertility effect from DEHP, although the antifertility effects observed in this study may also be a consequence of the mutagenic effects rendering the sperm incapable of fertilizing the ovum or for the fertilized ovum to develop.

In a parallel study not reported here, the effect of lower doses of DEHP on testicular structure and function did not show any changes in histopathological organization or macromolecular contents (nucleic acids and protein) of the tissue, suggesting that

increased fetal deaths are not a consequence of testicular atrophy. Alterations in the activity of certain mitochondrial and lysosomal enzymes of testicular tissue were, however, observed after treatment with DEHP to account for the changes in the functional ability of the reproductive system.

It has always been a point of argument in selecting doses of DEHP and/or the route of administration in experimental animals which stimulate conditions close to that of human exposure or which provide predictive toxicologic data. A subcutaneous administration would therefore justify the parenteral administration like IV without any possible direct contact with the organs of concern and would certainly be more practical to assess the toxicity of DEHP than oral route where most of the effects would be due to metabolites rather than DEHP itself. Undoubtedly the doses used in the present study are much higher than would be expected to reach the human system through biomedical devices. However, in view of the marked increase in the chronic toxicity and evident accumulation of DEHP in primates, usage of higher doses of DEHP in such short term studies may help in indicating possible risk of mutagenic effects in human beings exposed to relatively smaller doses over a longer period of time.

Further studies in fertility and mutagenic effects of DEHP and other phthalate esters are continuing and the results will be reported in subsequent publications.

REFERENCES

1. Guess, W. L., Jacob, J., and Autian, J. A study of polyvinyl chloride blood bag assemblies I. Alteration or contamination of ACD solutions. *Drug Intell.* 1: 120-127 (1967).
2. Jaeger, R. J., and Rubin, R. J. Plasticizers from plastic devices: extraction, metabolism, and accumulation by biological systems. *Science* 170: 460-461 (1970).
3. Autian, J. Toxicity and health threats of phthalate esters: review of literature. *Environ. Health Perspect.* 4: 3-26 (1973).
4. Lawrence, W. H., Malik, M., Turner, J. E., Singh, A. R., and Autian, J. A toxicological investigation of some acute, short-term and chronic effects of administering di-2-ethylhexyl phthalate (DEHP) and other phthalate esters. *Environ. Res.* 9: 1-9 (1975).
5. Jacobson, M. S., Kevy, S. V., and Grand, R. J. Effects of plasticizer leached from polyvinyl chloride on the subhuman primate: a consequence of chronic transfusion therapy. *J. Lab. Clin. Med.* 89: 10066-78 (1977).
6. Singh, A. R., Lawrence, W. H., and Autian, J. Teratogenicity of phthalate esters in rats. *J. Pharm. Sci.* 61: 51-55 (1972).
7. Lewandowski, M., Fernandes, J., and Chen, T. S. Assessment of the teratogenic potential of plasma-soluble extracts of diethylhexyl phthalate plasticized polyvinyl chloride plastics in rats. *Toxicol. Appl. Pharmacol.* 54: 141-147 (1980).
8. Singh, A. R., Lawrence, W. H., and Autian, J. Mutagenic and antifertility sensitivities of mice to di-2-ethylhexyl phthalate (DEHP) and dimethoxyethyl phthalates (DMEP). *Toxicol. Appl. Pharmacol.* 29: 35-46 (1974).